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Florence Roucher-Boulez[1,2,*], Delphine Mallet-Motak[1], Dinane Samara-Boustani[3], Houweyda Jilani[1], Asmahane Ladjouze[4], Pierre-François Souchon[5], Dominique Simon[6], Sylvie Nivot[7], Claudine Heinrichs[8], Maryline Ronze[9], Xavier Bertagna[10], Laure Groisne[11], Bruno Leheup[12], Catherine Naud-Saudreau[13], Gilles Blondin[13], Christine Lefevre[14], Laetitia Lemarchand[15], Yves Morel[1,2]

[1]Molecular Endocrinology and Rare Diseases, Lyon University Hospital, Bron, France;

[2]Claude Bernard Lyon 1 University, Lyon, France;

[3]Pediatric Endocrinology, Gynecology and Diabetology, Necker University Hospital, Paris, France;

[4]Pediatric Department, Bab El Oued University Hospital, Alger, Algeria;

[5]Pediatric Endocrinology and Diabetology, American Memorial Hospital, Reims, France;

[6]Pediatric Endocrinology, Robert Debré Hospital, Paris, France;

[7]Department of Pediatrics, Rennes Teaching Hospital, Rennes, France;

[8]Pediatric Endocrinology, Queen Fabiola Children's University Hospital, Brussels, Belgium;

[9]Endocrinology Department, L.-Hussel Hospital, Vienne, France;

[10]Endocrinology Department, Cochin University Hospital, Paris, France;

[11]Endocrinology Department, Lyon University Hospital, Bron-Lyon, France;

[12]Paediatric and Clinical Genetic Department, Nancy University Hospital, Vandoeuvre les Nancy, France;

[13]Pediatric Endocrinology and Diabetology, Bretagne Sud Hospital Center, Lorient, France;

[14]Pediatric Endocrinology, Jeanne de Flandre Hospital, Lille, France;

[15]Pediatric Department, La Rochelle-Ré-Aunis Hospital Group, La Rochelle, France.

***Corresponding author:**

Florence Roucher-Boulez

Laboratoire d'Endocrinologie Moléculaire et Maladies Rares,

Centre de Biologie et de Pathologie Est, 59 Bd Pinel, 69677 Bron Cedex, France

Phone: +33 (0)4 72 12 96 79

Fax: +33 (0)4 72 12 97 20

E-mail: florence.roucher@chu-lyon.fr

Short title: *NNT*, adrenal and extra-adrenal defects

Key words: nicotinamide nucleotide transhydrogenase, oxidative stress, primary adrenal insufficiency, massive parallel sequencing, follow-up

Word count:

Text word count: 3880

Abstract word count: 239

References: 42

Tables: 2

Figures: 4

ABSTRACT

***Objective*:** *NNT (nicotinamide nucleotide transhydrogenase)*, one of several genes recently discovered in familial glucocorticoid deficiencies (FGD), is involved in reactive oxygen species detoxification, suggesting that extra-adrenal manifestations may occur, due to the sensitivity to oxidative stress of other organs rich in mitochondria. Here, we sought to identify *NNT* mutations in a large cohort of patients with primary congenital adrenal insufficiency without molecular etiology and evaluate the degree of adrenal insufficiency and onset of extra-adrenal damages.

Methods: Sanger or massive parallel sequencing of *NNT* and patient monitoring.

Results*:* Homozygous or compound heterozygous *NNT* mutations occurred frequently (26%, 13 unrelated families, 18 patients) in our cohort. Seven new mutations were identified: p.Met337Val, p.Ala863Glu, c.3G>A (p.Met1?), p.Arg129*, p.Arg379*, p.Val665Profs*29, and p.Ala704Serfs*19. The most frequent mutation, p.Arg129*, was found recurrently in patients from Algeria. Most patients were diagnosed belatedly (8-18 months) after presenting severe hypoglycemia; others experiencing stress conditions were diagnosed earlier. Five patients also had mineralocorticoid deficiency at onset. One patient had congenital hypothyroidism and two cryptorchidism. In follow-up, we noticed gonadotropic and genitalia impairments (precocious puberty, testicular inclusions, interstitial Leydig cell adenoma, azoospermia), hypothyroidism and one hypertrophic cardiomyopathy. Intrafamilial phenotype heterogeneity was observed.

Conclusions*: NNT* should be sequenced, not only in FGD, but also in all primary adrenal insufficiencies for which the most frequent etiologies have been ruled out. As NNT is involved in oxidative stress, careful follow-up is needed to evaluate mineralocorticoid biosynthesis extent, and gonadal, heart and thyroid function.

INTRODUCTION

Primary adrenal insufficiency (PAI) is a life threatening disorder. Three types can occur: isolated mineralocorticoid deficiency, isolated glucocorticoid deficiency, or combined mineralocorticoid and glucocorticoid deficiency (global adrenal insufficiency). Glucocorticoid deficiencies, also called ACTH resistance syndromes, are autosomal recessive disorders. They include familial glucocorticoid deficiency (FGD) (OMIM#202200) and triple A syndrome (AAAS) (OMIM#231550), also known as Allgrove syndrome [1, 2]. Patients present episodes of hypoglycemia in the neonatal period or early childhood with low or unquantifiable cortisol, elevated ACTH levels and normal aldosterone and plasma renin measurements. Until 2012, only a half of FGD cases could be explained by homozygous or compound heterozygous mutations in genes involved in the steroidogenic pathway: *MC2R* (25%), *MRAP* (20%), *STAR* (5%) and more rarely *CYP11A1* [3-7]. Over the last three years, thanks to whole exome sequencing, three more causative genes have been discovered: *MCM4 (mini chromosome maintenance deficient 4 homologue), NNT (Nicotinamide Nucleotide Transhydrogenase)* and *TXNRD2 (Thioredoxin reductase 2)* [8-10].

As these genes encode proteins that work together for reactive oxygen species (ROS) detoxification or DNA replication, the spectrum of pathogenic mechanisms causing PAI is not limited to genes involved in adrenal development and steroidogenesis.

The incidence of *NNT* gene mutations in Clark et al's FGD cohort was around 10% (15 families) and no predominant mutation was reported (21 private mutations)[9]. Twelve more families have been reported (12 additional mutations), some with mineralocorticoid defects [11-16].

NNT encodes an integral protein of the inner mitochondrial membrane that acts as a proton pumping transhydrogenase [17]. In prokaryotic cells, the enzyme is composed of two or three different subunits, whereas in eukaryotic cells, it is usually composed of a single subunit. The active form of the enzyme is always a homodimer of approximately 220 kDa. All NNTs show a similar structure with three major domains. Domain I contains the hydrophilic NAD(H) binding site and domain III, the hydrophilic NADP(H) binding sites. Domain II constitutes the hydrophobic transmembrane part of the enzyme that connects domains I and III and forms the proton channel [18]. NNT supplies the high concentrations of NADPH needed for glutathione and thioredoxin antioxidant systems involving enzymes such as

GPX1 (glutathione peroxidase 1), TXNRD2 and PRDX3 (peroxiredoxin 3). NADPH is a cofactor of P450 enzymes, notably in steroidogenesis [19-22] **(Figure 1).**

Meimaridou *et al* showed reduced, basal and ACTH-stimulated corticosterone, revealing impaired steroidogenesis in C57BL/6J mice with a spontaneous *NNT* mutation (an in-frame 5 exon deletion). Furthermore, they showed increased ROS levels in an NNT knock-down human adrenocortical cell line [9]. Oxidative stress impedes steroidogenesis, which in turn induces more oxidative stress resulting from electron leaks throughout the steroidogenic pathway. Why it affects adrenal hormone production preferentially remains unknown. All tissues rich in mitochondria may be affected, resulting in a wide spectrum of diseases. Phenotypically, C57BL/6J mice do not have adrenal defects but show glucose intolerance and impaired insulin secretion [23]. At present, in humans, NNT mutations are known to be associated with adrenal insufficiency. Additionally, relationships between decreased NNT activity, modified mitochondrial redox regulation and cardiac failure have been recently reported [24-26].

The aim of our study was to screen for *NNT* mutations in fifty families with PAI with no identified molecular etiologies and to perform a careful follow-up so as to identify any extra-adrenal defects. We found thirteen families (eighteen patients) with *NNT* mutations: thirteen patients were diagnosed with FGD and five with global adrenal insufficiency at onset. A range of functions, i.e., adrenal/mineralocorticoid, puberty, fertility, heart, pancreatic, thyroid and growth, were subjected to long-term monitoring.

PATIENTS AND METHODS

Patients

The *NNT* gene was analyzed in fifty patients with primary adrenal insufficiency with no molecular diagnosis. Informed consent was provided by all enrolled patients and the study was conducted in accordance with the principles of the Declaration of Helsinki. Very long-chain fatty acids in boys and 17-hydroxyprogesterone in all patients were either within normal limits or low, excluding adrenoleukodystrophy and 21-hydroxylase deficiency, and adrenal autoantibodies were negative, excluding an autoimmune disorder. Mutations in *STAR*, *CYP11A1, MC2R* and *MRAP* were excluded by Sanger sequencing, as were those in *NR0B1* for boys.

Molecular genetic analysis of the *NNT* gene

Genomic DNA was extracted from EDTA-preserved whole blood using the Nucleon BACC3 kit (GE healthcare, Chalfont Saint Giles, Buckinghamshire, UK). Sanger sequencing was done for 47 patients and massive parallel sequencing (MPS) for three (patients 11, 12 and 13 in Table 1).

Sanger sequencing

Selective amplification of the 21 coding exons of the *NNT* gene was performed in twenty fragments by PCR using specific primers (available on request). Conventional dideoxy sequencing of exons and exon-intron boundaries was done using Big-Dye Terminators. Sequencing products were loaded on an ABI-3730XL and analyzed using SeqScape software v2.5 (Life Technologies, CA, USA). Sequence variants were designated according to the Human Genome Society recommendations (www.hgvs.org/rec.html) using the NCBI reference sequences NC_000005.9, NM_012343.3 and NP_036475 built on the GRCh37/hg19.

Massive parallel sequencing (MPS) or next generation sequencing (NGS)

DNAs were tested using an amplicon-based library preparation. A custom panel targeting 57 genes, involved in adrenal insufficiency and disorders of sex development, including *NNT*, was designed using Ion AmpliSeq designer software (Life Technologies) (coding regions ± 50bp) (article underway, list available on request). The library preparation was done according to the manufacturer's instructions with the Ion AmpliSeq Library Kit v2.0 (Life Technologies). Enrichment and quantification of target DNA were validated on the Caliper LabChip-GX using the high sensitivity

assay kit (Caliper LifeSciences Waltham, MA, USA). The patients were barcoded and pooled by groups of eight to get a sufficient depth of coverage (>100X) at sequencing. For the sequencing step, enriched template-positive Ion PGM spheres were prepared by emulsion PCR with the Ion OneTouch 2 System (Life Technologies). The resulting live Ion Sphere Particles (ISPs) were loaded on an Ion 316 Chip. Sequencing was done on the Ion Torrent Personal Genome Machine (PGM) with the PGM Sequencing 200 Kit. The bioinformatics pipeline used was the Torrent Suite software implemented with the sequencer and with the default parameters. *NNT* mutations were validated by Sanger sequencing.

Array comparative genomic hybridization (aCGH) and long range PCR

To confirm a deletion, aCGH or chromosomal microarray (CMA) was performed according to the manufacturer's instructions, using the Agilent SurePrint G3 Human CGH Microarray 4x180K AMADID 022060 (Agilent Technologies, Inc, Santa Clara, CA). This was followed by long-range PCR using the Qiagen LongRange PCR kit (Qiagen, Hilden, Germany) according to the supplier's recommendations. Conventional dideoxy sequencing of the PCR product was done as described in the paragraph "Sanger sequencing" (primers available on request).

Pathogenicity prediction

Multiple sequence alignment

Multiple sequence alignment of NNT protein sequences from different species was used to analyze structurally conserved regions and to predict putative effects of missense mutations. The sequences were found in the Uniprot database (http://www.uniprot.org/), aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default parameters, displayed and then edited using Genedoc (http://www.psc.edu/index.php/user-resources/software/genedoc).

Software and databases

For each new missense mutation, pathogenicity was predicted *in silico* using several programs: align GVGD, Polyphen 2, SIFT and Mutation taster. The Grantham score was calculated to predict the effect of substitutions between amino acids. This score looks at chemical properties to define a score range between 0 and 215. Higher scores indicate greater differences in the chemical properties between two amino acids (i.e., polarity and molecular volume) and may indicate a stronger (negative)

effect on protein structure and function. The dbSNP, EVS and ExAC browser databases were searched to determine if variants had already been reported.

RESULTS

NNT gene sequencing

Ten different *NNT* mutations, scattered throughout the gene, were found in thirteen families (eighteen patients) **(Table 1, Figure 2)**. Seven of them were new mutations: two nonsense (p.Arg129*, p.Arg379*), two missense (p.Met337Val, p.Ala863Glu), two frameshift (p.Val665Profs*29, p.Ala704Serfs*19), and one start loss (c.3G>A (p.Met1?)). The p.Arg129* mutation was found in four families, all of Algerian origin (**Table 2).** Consanguinity was present in eight of the thirteen families and homozygous mutations were found in eleven families. No consanguinity was found in the other homozygous families (1, 5 and 10), but the parents in family 10 were from the same small village in France. The patients of families 4 and 12 were compound heterozygotes. Patient 12 was first thought homozygous for the mutation p.Arg71*. However, the mother did not carry the mutation and thus a deletion was suspected and thereafter confirmed by aCGH analysis and long range PCR sequenced step by step. For the three patients studied by MPS, all variants found in other genes were benign.

Pathogenicity prediction (Table 1)

The p.Pro437Leu and p.Arg71* mutations have already been described[9, 15]. For the frameshift or nonsense mutations, the consequences should be premature truncated proteins or an absence of protein due to intervention of the nonsense-mediated decay system. The new mutation, c.3G>A (p.Met1?), affecting the translation initiation site, should switch this latter to an in-frame downstream methionine at codon 192. In the absence of its N-terminal part, the resulting NNT should be non-functional**.**

To predict the pathogenicity of missense mutations, multiple alignments of NNT proteins were done in order to locate the changed residue in the protein structure and identify conservation between species [18, 27] (**Figure 3**).

The p.Ala863Glu mutation is located in the transmembrane helix 14 (H14) of domain II and is highly conserved between species (**Figure 3**). H14 appears to indirectly facilitate proton translocation by influencing the centrally-located H9, H10, and H13, in which the proton channel is assumed to be located. In *Escherichia coli*, mutations of certain residues in these regions result in intermediate inhibitory effects [28]. This mutation may disrupt the conformational changes responsible for interconversion of the open and occluded states [29]. It may also play a role in coupling between the

redox state of the nucleotide and the proton movement in the protein, as it is near the NADP(H) binding domain. In *in silico* predictions, this mutation was most likely pathogenic using Align-GVGD class, probably damaging using Polyphen-2, deleterious using SIFT and disease causing using Mutation Taster. The high Grantham score of 107 is also concordant. This mutation is not reported in dbSNP, EVS or ExAC browser databases and has not been found in one hundred French Caucasian healthy controls. As expected, the parents of patient 7 were heterozygous as were his two healthy brothers.

The missense mutation, p.Met337Val, was identified in the NAD(H) binding domain near the NAD(H) binding site. It should inhibit the hydride transfer from NADH to $NADP^+$. The residue is highly conserved between species (**Figure 3**). The mutation was predicted to be deleterious by all of the mutation prediction tools mentioned above, despite a Grantham score of 21. This variation was not listed in the databases (dbSNP, EVS or ExAC browser) and not found in one hundred healthy controls from the Maghreb.

Clinical data at onset and follow-up (Table 2)

Table 2 presents the clinical data and follow up for the eighteen patients reported. Only the predominant features are presented in the text.

Clinical presentation at age of diagnosis

Severe hypoglycemia, sometimes leading to coma, was the main symptom at age of diagnosis in all but two patients (numbers 11a and 12). That symptom was often associated with infections and melanoderma. This latter, upon inquiry, was often reported to have been present before the hypoglycemia. Five out of the thirteen families had experienced multiple deaths of other children; although not diagnosed at the time, those deaths too were probably due to adrenal insufficiency and severe hypoglycemia. Patient 11a and 12 experienced salt wasting (SW) at onset without hypoglycemia and three other patients had a global adrenal insufficiency (patients 2b, 6, 8) with SW. The median age at onset in our cohort was 11.5 months (min-max: 1.5 months-4 years) **(Table 2, Figure 4A)**. Most cases were diagnosed belatedly around the first year of life (8-18 months) but some involving stress conditions were diagnosed earlier. A difference in age at onset was detected between the subgroup with isolated glucocorticoid deficiency and that with global adrenal insufficiency. This

was the case for both our cohort alone (Kruskal-Wallis test: p-value =0.03379, **Figure 4B)** and our cohort aggregated with the data available in the literature (Kruskal-Wallis test: p-value =0.003705, **Figure 4 C**). However, no difference in age at onset was found between the subgroup homozygous for non-truncated mutations and that homozygous for truncated mutations (Kruskal-Wallis test: p-value =0.2172).

Follow-up

At study end, the age of the 16 patients ranged from 4 to 57 years old, permitting a long patient follow-up.

- Mineralocorticoid function:
    - Patient 3 had SW at age 15 then recurrence at 18, illustrating the importance of follow-up. Moreover, eight other patients (1, 2a, 4a-b, 10a-c, 13) had elevated renin and/or low aldosterone and needed mineralocorticoid or salt therapy.
- Gonadotropic/genitalia function:
    - Patients 11a and 11b, both presented with cryptorchidism and underwent surgery for ectopic testes.
    - Two patients (7 and 9) had precocious puberty at age five, associated with testicular nodules, low or undetectable gonadotropins and high testosterone. Patient 9 had surgery revealing an interstitial Leydig cell adenoma. In this patient, a short GnRH analog therapy was discontinued and the adenoma removed. Thereafter, testosterone remained at pre-pubertal values.
    - For patient 12, testicular inclusions were detected at age 18 during imaging studies for azoospermia and were consistent with testicular adrenal rest tumor (TART). His azoospermia was associated with elevated FSH (LH: 9 mUI/ml, FSH: 18 mUI/ml) but normal testosterone (5.8 nmol/L). His karyotype was normal (46,XY) with no Y chromosome microdeletion. Increasing the dose of his glucocorticoid replacement therapy did not reduce the testicular inclusion and had no effect on spermatogenesis.
    - Patient 1 had a testicular biopsy at age 31 for a left varicocele with epididymitis.
- Heart function:

- A transthoracic echocardiography in patient 1, at age 23 showed a typical and severe asymmetrical left ventricular hypertrophy (maximal wall thickness measured at the basilar septum: 36 mm). The resting left ventricular outflow gradient was measured at 15 mmHg. There was no mitral regurgitation. The left atrium was dilated (25 mm²). Patient 9 at age six had normal heart function but with a left ventricular ejection fraction of 75%.

- Other functions:
  - Two patients (2b and 9) had hypothyroidism with a thyroid gland in place. Patient 2b had congenital hypothyroidism and patient 9 hypothyroidism with low free T4 at age five and elevated TSH with no goiter at age seven.
  - Three patients had recurrent urinary tract infections (4b, 11a, 13).
  - We did not have information on social aspects for all the patients but four of them (patients 3, 5, 7 and 9) were experiencing poor academic performance or acquisition delays, possibly due to severe hypoglycemia.
  - None of the patients presented pancreatic dysfunction or impaired glucose tolerance. There were no growth disorders for patients who reached adult age.

DISCUSSION

Here, we report seven new *NNT* mutations identified in eighteen patients, eleven with FGD and seven with global adrenal insufficiency, who were members of thirteen families, i.e., 26% of the fifty families studied.

The mutations were distributed throughout the gene and most led to a premature truncated protein or an absence of protein. The most frequent mutation found in our cohort was p.Arg129*, which was identified in four Algerian families, suggesting the possibility of a founder mutation similar to p.Gly200Ser in Palestine [12]. We also identified two novel missense mutations, which should be pathogenic. Our patients with NNT mutations displayed a severe phenotype, with adrenal insufficiency often revealed by hypoglycemic convulsions. Most of the cases were diagnosed belatedly, around the first year of life (8-18 months). Some however were discovered earlier if stress conditions had occurred, i.e., intercurrent infections, suggesting the need of a stress to trigger the disease (**Figure 4A**). This is in accordance with the literature where the minimum age at diagnosis is three days [12] and the median age at onset is 12 months for the 29 patients for whom data are available [9, 11-13, 15, 16]. Unlike those of Jazayeri *et al*, our data suggest earlier onset in patients with global adrenal deficiency compared to those with isolated glucocorticoid deficiency (**Figure 4B and C**). The phenotypic variability between patients having a same mutation or within a same family (family 2), suggests that there is no correlation between genotype and phenotype.

It is clear that NNT mutations can result in global adrenal deficiency. In the literature and comparably to fourteen of our patients, five families were recently described with mineralocorticoid deficiency present at onset and three others with elevated renin or electrolytes imbalance [11-13, 16]. Our patient 3 had salt wasting at age 15, although aldosterone requirements normally decrease throughout life [30]. We observed phenotypic heterogeneity even within a same family. Patient 11a presented salt wasting whereas patient 11b had no mineralocorticoid deficiency. This emphasizes the need for careful monitoring of this function, since some patients classified as FGD may also have a slight mineralocorticoid defect. It has been shown that C57BL6/J mice carrying *NNT* mutations have disorganized zonae fasciculata with higher levels of apoptosis [9]. As aldosterone requirement decreases

299 through life, the mineralocorticoid defect may be the consequence of extended damage to all adrenal
300 zona.
301 NNT has a role in the oxidative stress response and mutations in it may thus affect all tissues rich in
302 mitochondria. For this reason too, patients with NNT mutations need to be closely monitored. Two *in*
303 *vitro* studies on the fibroblasts [12] and lymphocyte mitochondria [31] of patients homozygous for missense
304 *NNT* mutations showed an increase in ROS levels, a decrease of ATP content, and impaired
305 morphology of mitochondria with reduced mitochondrial mass and increased mtDNA deletion due to a
306 lack of thymidylate biosynthesis [12, 31]. The results from those two studies suggest that all tissues can be
307 injured, as do our results from the follow-up of patients with extra-adrenal defects.
308 Although NNT is widely expressed in adrenal, heart, kidney, thyroid and adipose tissues, the most
309 affected tissue in our cohort appeared to be the gonads [9]. Two of our patients had cryptorchidism
310 (patients 11a and b) and two others (7 and 9) presented similar histories involving, both at about five
311 years of age, the development of palpable nodules on the testicular surface or testicular enlargement
312 followed by the onset of puberty with high testosterone levels. These last two cases are comparable to
313 that reported by Hershkovitz *et al* [13]. For our patients 7 and 9, gonadotropins were in the normal pre-
314 pubertal range and the increase in testosterone seemed to be due to secretion by autonomous nodules
315 responsible for the onset of puberty. Since the regression of puberty for patient 9 was due either to the
316 removal of the adenoma or the short GnRH analog treatment, we cannot pronounce as to the central,
317 peripheral or mixed origin of the precocious puberty. Reporting on a boy with a mutation in DAX-
318 1/NR0B1, Domenice *et al* concluded that chronic excessive ACTH levels may stimulate Leydig cells
319 and lead to gonadotropin-independent precocious puberty [32], a view toward which Hershkovitz et al's
320 case [13] argues as well. In contrast, the testicular inclusions of our patient 12, associated with
321 azoospermia but normal testosterone values at age 18, although not reduced by glucocorticoid therapy,
322 should be TART, often found in congenital adrenal hyperplasia [33].
323 To date, we have observed heart function impairment (progressive hypertrophic cardiomyopathy) in
324 only one of our patients but cannot exclude future cases because of the mean age of our cohort and the
325 subnormal imaging of patient 9. This underlines the specific role of NNT in heart tissue. In B6J-Sod2-
326 /- mice, the presence of a normal *NNT* allele preserves cardiac function, delays the onset of heart

failure, and extends survival to the end of gestation [24]. In comparison, the suppression of *NNT* in zebrafish results in ventricular malformations and contractile dysfunctions [34]. Moreover, in humans, relationships between decreased NNT activity, modified mitochondrial redox regulation and cardiac failure have been reported. In the failing human heart, a partial loss of NNT activity adversely affects NADPH-dependent enzymes and the capacity to maintain membrane potential. This contributes to a decline in bioenergetic capacity, redox regulation and antioxidant defense, exacerbating oxidative damage to cellular proteins [26]. A recent report of a heterozygous frameshift mutation of *NNT* in humans with left ventricular noncompaction supports the assumption that NNT plays a major role in myocardium [34]. However, Nickel *et al* demonstrated a completely opposing view. They reported that during heart pressure overload, NNT adopts a reverse mode contributing to oxidative stress from which mice with mutation in *NNT* are protected [35]. Those puzzling new insights may suggest that the functional mode (forward or reverse) of NNT is dependent on the metabolic state. Nevertheless, TXNRD2 is in the same pathway of ROS detoxification and *TXNRD2* heterozygous mutations in humans have also been linked to dilated cardiomyopathy. Thus, for now, cardiac follow-up should be done [10, 36].

The thyroid gland, highly exposed to oxidative stress, was the third most-affected organ in our cohort. Beyond our two patients with hypothyroidism, probably due to some hormone synthesis defect, two other cases with subclinical hypothyroidism have been reported [16]. The biosynthesis of thyroid hormones (TH) is an oxidative biochemical reaction that depends on the formation of peroxide. However, two studies have suggested that when thyroid cells are exposed to significant amount of ROS, thyroid peroxidase and iodide organification are inhibited [37, 38]. Another argument is the prevalence of thyroid dysfunction in patients with Down syndrome who are under unusual increased oxidative stress [39]. NNT mutations may disturb the balance between H_2O_2 produced for TH biosynthesis and anti-oxidants to protect cells from H_2O_2 mediated oxidative damages, thus leading to TH formation inhibition. Nevertheless, our patients with hypothyroidism were consanguineous and we cannot exclude that another gene involved in the thyroid may be mutated.

Other functions in our patients were normal, especially growth and glucose metabolism. Glucose intolerance or diabetes in humans has not been reported in the setting of NNT mutation, although

defects in mitochondrial energy metabolism have also been implicated in diabetes. This contrasts with the impaired insulin secretion observed in *NNT* mutant mice for which only the β–cells seemed sensitive [40]. Increased ROS usually plays a role in innate immunity against bacterial cyto-invasion [41]. Despite that, three of our patients experienced recurrent urinary tract infections. We thus feel that additional studies are necessary to further investigate renal function.

In conclusion, we report here mutations in the *NNT* gene, which was one of the most frequent molecular etiologies in our "atypical" congenital adrenal insufficiency cohort. Deducing from our results and those of other authors, mutations in *NNT* should be searched not only in FGD but also in global adrenal insufficiency. Above all, careful follow-up, especially for mineralocorticoid, puberty, fertility, heart and thyroid function, must be maintained for all patients. The MPS approach described in the methods section, with a large panel of genes including *NNT*, appears to be ~~the most~~ efficient for genetic diagnosis [16, 42]. The analysis of more than one gene at a time is a powerful way to reach a diagnosis in diseases with phenotype heterogeneity. We note that more and more "atypical" cases of PAI are being described, for example STAR and CYP11A1 mutations in boys with PAI with or without DSD.

DISCLOSURE

Declaration of interest

The authors declare that there are no conflicts of interest that could prejudice the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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TABLE AND FIGURES LEGENDS

Table 1: NNT mutations in thirteen families with PAI. d: domain, TMH: transmembrane helix, -: not applicable, NMD: nonsense-mediated decay

Table 2: Clinical characteristics and follow-up of the patients with NNT mutations. -: not determined, M: male, F: female, Nl: normal,*: deceased, yrs: age in years, ttt: treatment, MC: mineralocorticoid, GC: glucocorticoid, SW: salt wasting, ↘: decreased ↗: increased, LVEF: left ventricular fraction, ENT: ear, nose, throat. Major abnormalities are in bold. For two patients (4a and 9) the age of diagnosis was late despite an earlier onset.

Figure 1: Role of NNT in free radical metabolism in the mitochondria ETC: electron transport chain GSSG: glutathione disulfide; GSH: glutathione; GPX: glutathione peroxidase; GR: glutathione reductase; TXNRD2: thioredoxin reductase; PRDX3: peroxiredoxin 3

Figure 2: NNT Mutations. Comparison between the domain structure of NNT protein and *NNT* exons in humans. Above: nonsense or frameshift mutations; below: missense mutations. New mutations indicated by rectangles. Underlined mutation is probably a splicing mutation.

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Figure 3: Partial multiple amino acid alignment of NNT in human, bovine, mouse, *Caenorhabditis elegans*, *Escherichia coli* and *Acetabularia acetubulum*. Alignment was performed in clustalW and edited with Genedoc. The mutant residues p.M337V and p.A863E and corresponding amino acids are shaded and show the conservation across all species.

Figure 4: A. Age at presentation for the patients of our cohort GD: glucocorticoid deficiency, SW: salt wasting. **B&C**. Difference in age at presentation (Kruskal-Wallis test, *: significance at $p<0.05$) between the group of patients with glucocorticoid deficiency and the group with salt wasting +/-

glucocorticoid deficiency within our cohort (p-value =0.03379*) **(B);** our data aggregated with the data available in the literature (p-value =0.003705*) **(C)**.

Table 1 : NNT mutations in thirteen families with PAI

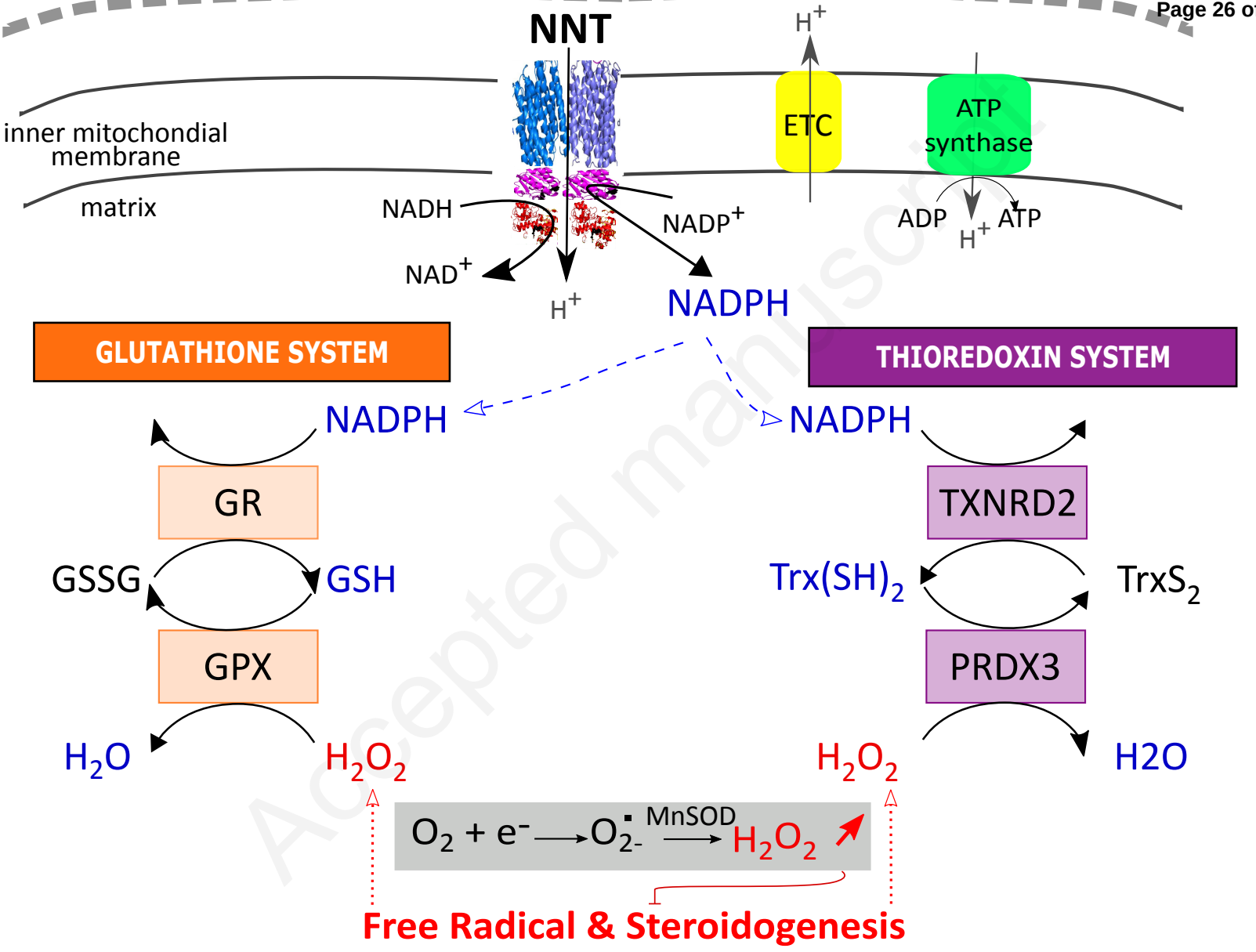
Nucleotide change (NM_012343.3)	Exon	Protein change (NP_036475)	Protein consequence	Domain	Predictive software				dbSNP ID	Allele count		Family number	Ref.
					GVGD	SIFT	Polyphen 2	Mutation Taster		ESP	ExAC		
c.3G>A	2	p.M1?	Start loss	Pre-sequence					-	0/13006	0/121286		9,14
c.211 C>T	3	p.R71*	Premature truncation at amino acid 71. NMD?	dI					-	0/13006	0/121286	1,4,12	15
c.385 C>T	4	p.R129*	Premature truncation at amino acid 129. NMD?	dI					-	0/13002	0/121286	2,3,4,11	
c.1009A>G	8	p.M337V	Missense mutation at amino acid 337 in the -DH binding domain protein	dI	less likely	Deleterious	Probably damaging	Disease causing	-	0/13006	0/121286	13	
c.1135C>T	9	p.R379*	Premature truncation at amino acid 379. NMD?	dI					-	0/13006	0/121286	9	
c.1310C>T	10	p.P437L	Missense mutation at amino acid 437 Role in dI-dII/dIII communication	dI	less likely	Deleterious	Probably damaging	Disease causing	-	0/13006	1/120146	10	9
c.1992_2005del	14	p.V665Pfs*29	Frameshift: premature truncation at amino acid 694	dII TMH7					-	0/13006	0/121286	5	
c.2106_2109dup	15	p.A704Sfs*19	Frameshift: premature truncation at amino acid 723	dII TMH9					-	0/13006	0/121286	6	
c.2588C>A	17	p.A863E	Missense mutation at amino acid 863 in the transmembrane domain (helix 14)	dII TMH14	Most Likely	Deleterious	Probably damaging	Disease causing	-	0/13006	0/121286	7	
c.(-51+1_-53-1)_(381+1_382-1)del	2-3	p.0?	Start loss. Absence of protein								0/121286	12	

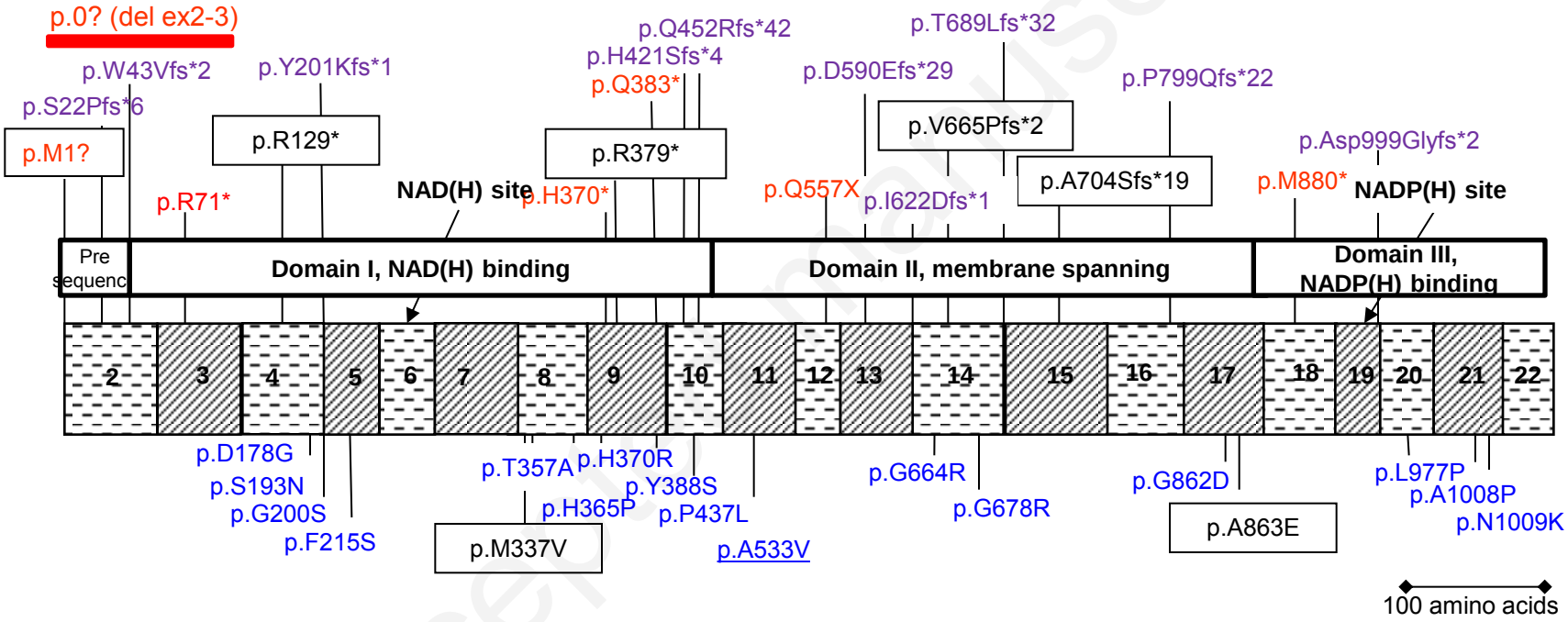
d:domain, TMH: transmembrane helix, -: not applicable, NMD nonsense-mediated decay

Table 2: Clinical characteristics and follow-up of the patients with *NNT* mutations.

Family	Origin	Consan-guinity	NNT mutation NP_036475	Sex	Age (yrs)	Age at diagnosis (months or yrs where indicated)	Clinical data at age of diagnosis	Mineralocorticoid defect	Gonads	Heart	Thyroid	Related death
1	French	no	p.R71*/ p.R71*	M	43	14	hypoglycemia, melanoderma	thirst for salt, MC ttt (↗renin)	onset of puberty at 12 yrs, Nl testicular function, **surgery for varicocoele**	**hypertrophic myocardiopathy**	Nl	brother at 2 yrs
2a	Algerian	yes	p.R129*/ p.R129*	M	32	6	hypoglycemic convulsions, delay of diagnosis due to GC therapy for asthma	MC ttt at 3 yrs (↗ renin)	onset of puberty at 13 yrs 9 months	-	Nl	
2b				M	15	3	hypoglycemic convulsions, **SW**	MC ttt, SW at 12 yrs after an attempt to stop MC ttt	onset of puberty at 12 yrs, low testosterone at 15 yrs	Nl imaging (12 yrs)	**congenital hypothyroidism** with thyroid gland in place	sister at 7 months
3	Algerian	yes	p.R129*/ p.R129*	F	19	15	hypoglycemia, asthenia, melanoderma	no MC ttt but 4 adrenal crises with ↗ renin ↘aldosterone (4, 7, 8 and 10 yrs) **SW** at 15 and 18 yrs	menarche (11 yrs), Nl menstrual cycle	Nl imaging (15 yrs)	Nl	no
4a	French/ Algerian	no	p.R71*/ p.R129*	F	30	1.5 (onset) /12	hypoglycemic convulsions following gastroenteritis	MC ttt at 2 yrs (↗ renin)	menarche (11 yrs), two children	-	-	no
4b				F	23	10	hypoglycemic convulsions following gastroenteritis	no MC ttt but ↗ renin	-	-	-	no
5	French	no	p.V665Pfs*29/ p.V665Pfs*29	M	14	19	hypoglycemic convulsions on ENT infections	no	onset of puberty at 12 yrs low testosterone at 13 yrs	Nl imaging (11 yrs)	Nl	no
6	Turkish	yes	p.A704Sfs*19/ p.A704Sfs*19	F	18	8	hypoglycemia, asthenia, melanoderma, weight loss, fever, **SW**	MC ttt	menarche (12.5 yrs)	-	**transient subclinical hypothyroidism** (TSH: 6.8 mUI/L at 5yrs)	no
7	Moroccan	yes	p.A863E/ p.A863E	M	5	13	hypoglycemic convulsions	no	**precocious puberty with 3 nodular testis**	Nl imaging (4 yrs)	Nl	no
8	Mauritian	yes	p.M1?/p.M1?	F	8	9	hypoglycemic coma, **SW**	MC ttt	no pubertal symptoms	-	Nl	no
9	Algerian	yes	p.R379*/ p.R379*	M	10	22 (onset) /8yrs	Misdiagnosed at 22 months (several hyperthermic convulsions, psychomotor retardation, sodium valproate ttt), all symptoms improved after glucocorticoid therapy initiated at 8 yrs	no	**Leydig cell adenoma (5 yrs) following by precocious puberty**	subNl imaging at 6 yrs **(LVEF at 75%)**	**subclinical hypothyroidism** (TSH: 3.5 mUI/L at 5 yrs and 10.5 at 7 yrs), thyroid hormone treatment	no
10a	French	no	p.P437L/ p.P437L	M	57	4 yrs	melanoderma, asthenia	salt craving, MC ttt	Nl puberty	-	-	no
10b				M	51	18	melanoderma, asthenia	salt craving, MC ttt	Nl testicular function at 49 yrs	Nl imaging (51 yrs)	Nl	no
10c				F	4*	16	familial story, deceased at 4 yrs	MC ttt	-	-	-	no
11a	Algerian	yes	p.R129*/ p.R129*	M	6	2	**SW**	SW (2 months, 4 yrs), MC ttt	**cryptorchidism (surgery)**	Nl imaging (2 yrs)	-	Brother at 8 months
11b				M	8	4 yrs	hypoglycemia (Nl at 3 yrs)	no	**cryptorchidism (surgery)**	Nl imaging (2 yrs)	-	Brother at 8 months
12	French	no	p.R71*/ del ex2-3	M	35	10	**SW**	SW, MC ttt	**bilateral TART, azoospermia**	-	Nl	no
13	Algerian	yes	p.M337V/ p.M337V	F	9	8	melanoderma, asthenia	i↗ renin,↘ aldosterone (16 months, 12 yrs), salt , no MC ttt	no pubertal symptoms	-	Nl	Sister at 4 yrs

-: not determined, M: male, F: female, Nl: normal,*: deceased, yrs: years, ttt: treatment, MC: mineralocorticoid, GC: glucocorticoid, SW: salt wasting, ↘: decreased ↗: increased, LVEF: left ventricular fraction, ENT: ear, nose, throat. Major abnormalities are in bold. For two patients (4a and 9) the age of diagnosis was late despite an earlier onset.





p.M337

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Human          :  VLFNKEMIESMKEGSVVVDLAAEAGGNFET
Bovine         :  ILFNKEMIESMKEGSVVVDLAAEAGGNFET
Mouse          :  VLFSKEMIESMKEGSVVVDLAAEAGGNFET
C. elegans     :  ILITEEMIKSMKPGSVVVDLAAESGGNIAT
E. coli        :  KLITREMVDSMKAGSVIVDLAAQNGGNCEY
A. acetabulum  :  KLILKDMIESMKPGSVVVDLAAENGGNIET
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p.A863

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Human          :  EGFLLNNNLLTIVGALIGSSGAILSYIMCV.
Bovine         :  EGFLLNNNLLTIVGALIGSSGAILSYIMCV.
Mouse          :  EGFLLNNNLLTIVGALIGSSGAILSYIMCV.
C. elegans     :  EGFMLDNSLLTVLGALIGSSGAILSHIMCK.
E. coli        :  AGFMLSNDLLIVTGALVGSSGAILSYIMCK.
A. acetabulum  :  G-SVLDNNLLTIVGALIGSSGAILSAIMCK.
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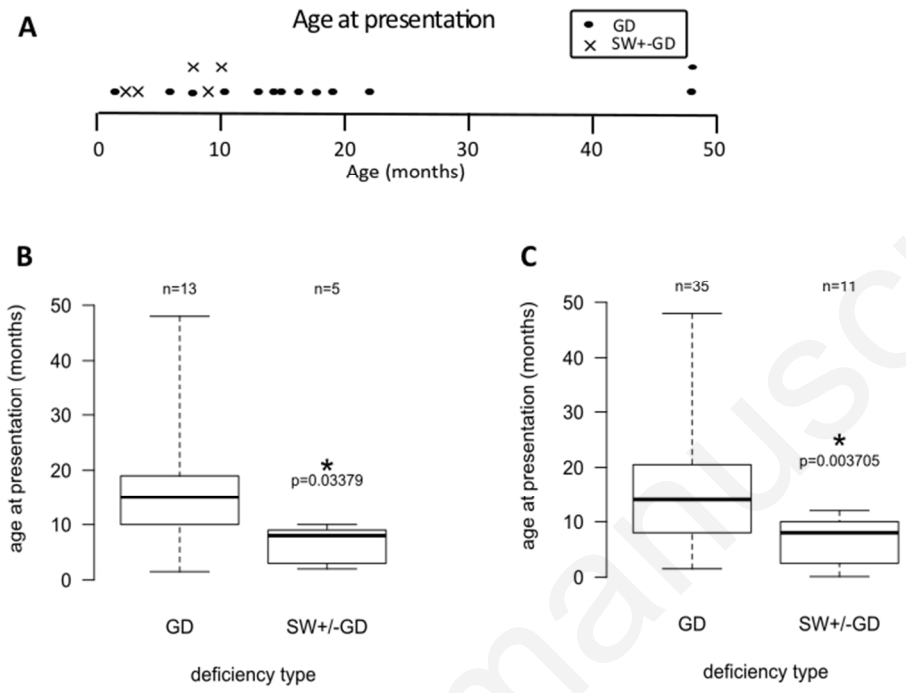

Figure 4: A. Age at presentation for the patients of our cohort GD: glucocorticoid deficiency, SW: salt wasting. **B&C**. Difference in age at presentation (Kruskal-Wallis test, *: significance at p<0.05) between the group of patients with glucocorticoid deficiency and the group with salt wasting +/- glucocorticoid deficiency within our cohort (p-value =0.03379*) **(B)**; our data aggregated with the data available in the literature (p-value =0.003705*) **(C)**.
67x50mm (300 x 300 DPI)